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Uncoupler-Resistant Mutants of Bacteria

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INTRODUCTION

The action of uncouplers on oxidative phosphorylation and other bioenergetic work that depends upon the energized state of the coupling membrane is most satisfyingly described by Mitchell's chemiosmotic hypothesis (68, 69). According to this formulation, the energized state of the membrane is an electrochemical proton gradient, $\Delta\bar{\mu}_{H^+}$, that is established by primary proton translocation events such as occur during respiration. The $\Delta\bar{\mu}_{H^+}$ can then be utilized and discharged during the function of the proton-translocating ATP synthase, H^+/solute symporters, and other catalysts of bioenergetic work located in the same membrane. Agents that directly dissipate the $\Delta\bar{\mu}_{H^+}$, e.g., by permeabilizing the coupling membrane to movement of protons or compensatory ions, short-circuit energy coupling and inhibit bioenergetic work while respiration (primary proton translocation) continues apace. That uncouplers do function in this manner has been extensively confirmed. For example, widely used protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) are weak lipophilic acids, whose uncoupling action correlates with their ability to transport protons down their electrochemical gradient (38, 40, 63, 79, 104). The protonophore cycles within the membrane in its protonated and deprotonated forms (67, 107). Kinetic models that describe proton transport across lipid bilayers agree well with data obtained from charge-pulse and voltage clamp experiments in which the effects of CCCP are examined at various pH values and voltages (51).

Bacteria might acquire resistance to uncouplers upon mutation by becoming able to exclude or inactivate given chemical classes of uncouplers. Alternatively, uncoupler-resistant mutants may still be sensitive to the effect of the uncouplers on the $\Delta\bar{\mu}_{H^+}$, but may have altered properties that allow bioenergetic work to proceed at unusually low $\Delta\bar{\mu}_{H^+}$ values. The former kind of mutant might be of pharmacological interest with respect to bacterial resistance to antibiotic compounds. The latter kind, if they can rigorously be shown to have unaltered susceptibility to the effect of the uncouplers upon the $\Delta\bar{\mu}_{H^+}$, are of considerable bioenergetic interest. At least at first blush, their retention of bioenergetic work capacity at submaximal values of the $\Delta\bar{\mu}_{H^+}$ appears to represent a departure from the chemiosmotic formulation. In that formulation, the steady-state level of bioenergetic work, represented by, for example, the phosphorylation potential, ΔG_p, is in direct and complete equilibrium with the $\Delta\bar{\mu}_{H^+}$, and the $\Delta G_p/\Delta\bar{\mu}_{H^+}$ ratio simply reflects the number of H^+ ions translocated per ATP molecule synthesized (17, 25, 26, 55). It is not trivial to rigorously demonstrate that a resistant mutant strain is still fully sensitive to the effect of the agent on the $\Delta\bar{\mu}_{H^+}$. Even in simple artificial systems, protonophoric activity, for example, is affected by many factors, such as buffering capacity of the aqueous phases, lipid composition and charge (including effects of surface charge on penetrability), bilayer thickness, and the membrane dielectric constant (67).

We will review the mutants that have been described previously, emphasizing the criteria and state of evidence for the existence of those of bioenergetic interest. As yet, there are no uncoupler-resistant mutants that can be shown to have inactivated the uncoupler, but there are clearly strains of *Escherichia coli* whose resistance arises from exclusion of the uncoupler from the energized coupling membrane. Among *Bacillus* species, in which the selective pressure is probably more straightforward because of the presence of a single membrane, mutants have been described whose $\Delta\bar{\mu}_{H^+}$ retains sensitivity to abolition by uncouplers but which perform bioenergetic work disproportionately well with respect to the residual force. The bioenergetic conundrum that this group presents depends upon the assumption of the Mitchellian understanding of uncoupler action. Therefore, we will also review observations that may diverge from that understanding and proposals for other modes of action of uncouplers that could alter the interpretation of mutant phenotypes. Finally, having discussed the uncoupler-resistant mutants of bacteria that have been reported to date, we

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will note a number of related or similar observations in other experimental systems.

MUTANTS OF *BACILLUS* SPECIES

Bacillus megaterium C8 Mutant

Strain C8, a spontaneously arising mutant strain, was isolated by Decker and Lang (16) from *Bacillus megaterium* on plating medium containing malate and 5 μM CCCP and was later studied in our laboratory as well. The mutant exhibited several bioenergetic changes. Malate-driven ATP synthesis in starved cells was resistant to CCCP and several other uncouplers including 2,4-dinitrophenol (DNP), pentachlorophenol (PCP), and salicylanilide. The respiratory rate of whole cells of mutant C8 in the presence of malate was approximately double that of wild-type cells (16, 26). The hydrolytic activity of ATPase in the mutant was only 10 to 25% of the wild-type activity (17, 26). Nonetheless, the rate and level of ATP synthesis in the two strains were comparable in the absence of inhibitors, with the measured $\Delta\bar{\mu}_{H^+}$ being the same or just slightly higher in the mutant (17, 26). Dicyclohexylcarbodiimide (DCCD) inhibited this synthesis in whole cells (17, 26) and vesicles (28) of both strains. In fact, C8 was somewhat more sensitive to DCCD than the wild type was (58). However, Decker and Lang (17) also reported the as yet unexplained observation that the effect of DCCD (or valinomycin) on malate-driven ATP synthesis by C8 cells could be reversed by CCCP. They proposed that a nonchemiosmotic route existed for energy coupling to ATP synthesis, perhaps involving a chemical intermediate; they noted that the resistance of C8 to uncouplers was consistent with proposals for a binding protein for such agents (discussed below), whose mutational change in C8 might lead to various elements of the bioenergetic phenotype (17).

Most of the observations of Decker and Lang (16, 17) have been confirmed in our laboratory, although under experimental conditions that used more dilute and highly aerated cell suspensions the resistance to protonophore was more modest than originally reported (16, 17). The outstanding characteristic was, again, the ability of C8 to synthesize substantial amounts of ATP at uncoupler-reduced $\Delta\bar{\mu}_{H^+}$ levels so low that they would not support ATP synthesis by the wild type (26). Starved whole cells of C8 generated the same transmembrane electrical potential, $\Delta\psi$ (at a pH at which the transmembrane pH gradient, ΔpH, was zero), and synthesized the same amount of ATP as did comparable wild-type cells upon addition of malate (Table 1). By contrast, C8 synthesized about four times more ATP than the wild type did upon addition of malate in the presence of enough CCCP (2 μM) to lower the re-established $\Delta\psi$ to −60 mV; under these conditions of submaximal $\Delta\bar{\mu}_{H^+}$, C8 exhibited a larger $\Delta G_p/\Delta\bar{\mu}_{H^+}$ (the ratio that is taken as the H^+/ATP stoichiometry in the chemiosmotic formulation) than the wild type did. Since the untreated cells generated comparable driving forces and synthesized comparable amounts of ATP upon reenergization, there was no inherently greater driving force or inherently greater H^+/ATP stoichiometry in the mutant at maximal $\Delta\bar{\mu}_{H^+}$ levels. Moreover, since CCCP reduced the driving force identically in the two strains, resistance did not arise from exclusion or inactivation of the uncoupler. Furthermore, similar resistance could be demonstrated in a vesicle system in which the electron donor was ascorbate-phenazine methosulfate; thus, the mutant phenotype could not be explained by some novel utilization of malate.

TABLE 1. $\Delta G_p/\Delta\psi$ ratio of *B. megaterium* and its uncoupler-resistant mutant derivative, C8, as a function of the mode of energization and the presence of CCCP[a]

Energization agent[b]	Addition	$\Delta\psi$[c] (mV)	ΔGp/Δψ for:	
			Wild type	C8
L-Malate	None	−120	3.4	3.3
	2 μM CCCP	−60	5.7	6.1
	5 μM CCCP	−38 to −50	6.4	7.0
Diffusion potential	None	−177	2.1[d]	1.9[d]
	None	−150	2.4	2.2
	None	−110	3.0	0[e]

[a] Data were compiled from reference 26 with permission of the publisher.
[b] Starved whole cells were energized by malate addition or a valinomycin-K^+ diffusion potential (26).
[c] $\Delta\psi$ was measured with [^{3}H]TPP$^+$.
[d] Diffusion potential-driven ATP synthesis was completely inhibited in both strains by either 2 or 5 μM CCCP.
[e] No ATP was synthesized.

Importantly, when ATP synthesis by starved cells was energized by an artificial $\Delta\bar{\mu}_{H^+}$, the mutant C8 did not function better than the wild type and synthesis was just as CCCP sensitive as in the wild type. Decker and Lang (17) had observed this for acid pulse-driven ATP synthesis; later, we conducted a series of experiments using valinomycin-mediated K^+ diffusion potentials (26). Diffusion potentials of various magnitudes (with measured values agreeing well with the calculated potentials) were generated across cyanide-treated starved cells. Both the wild type and C8 synthesized less ATP upon imposition of a large diffusion potential than had been observed upon generation of a comparable $\Delta\psi$ via respiration. Even more strikingly, C8 synthesized ATP less well at intermediate (−110 to −150 mV) diffusion potentials than did the wild type (Table 1). Moreover, under these conditions ATP synthesis by both strains was completely sensitive to 2 μM CCCP. These observations reinforced the conclusions that CCCP is equally active as a protonophore in both strains and that the phenotype of the mutant could not be explained by a capacity to utilize a larger H^+/ATP stoichiometry at lower $\Delta\bar{\mu}_{H^+}$ values. The latter should have allowed enhanced synthesis at a given submaximal diffusion potential, just as was observed with respiration. The observations further ruled out the possibility that the lower ATPase activity of the mutant was somehow crucially involved in the energetics or that unusual intracellular levels of P_i or ADP were involved; either would have obtained with both modes of energization, whereas the advantage of the mutant was manifest only when respiration was the energy source.

Studies of revertants and reversion frequency supported the view that the C8 phenotype resulted from a single mutation. After finding (as described in the next section) that comparable mutants of *Bacillus subtilis* are membrane lipid mutants that have elevated saturated/unsaturated fatty acid ratios in their membrane phospholipids, we examined the membrane lipids of the *B. megaterium* pair. Whereas the wild-type strain possessed 3% $isoC_{16:1}$, 7% $nC_{16:1}$, 3% $isoC_{17:1}$, and 5% $nC_{18:1}$ in its total membrane fatty acid content, the unsaturated fatty acid content of C8 was reduced to only 3% $nC_{16:1}$ (10). When the wild type was grown on medium supplemented with stearic acid, the saturated-to-unsaturated fatty acid ratio of this strain increased and a concomitant increase in CCCP resistance of growth was observed. Conversely, when the C8 strain was grown in

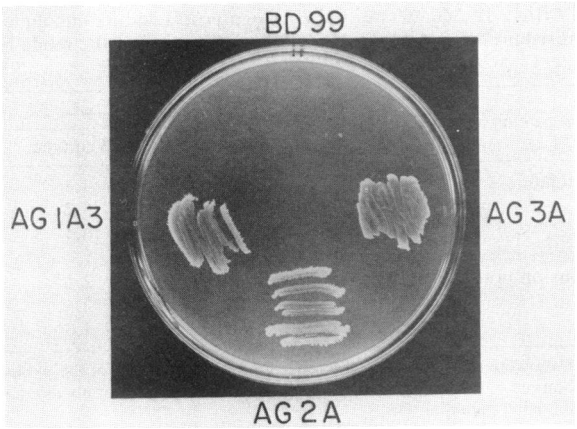

FIG. 1. Growth of wild-type *B. subtilis* BD99 and mutants AG1A3, AG2A, and AG3A on CCCP-containing medium. Logarithmically growing strains were streaked onto plating medium containing 50 mM DL-malate, 0.1% (wt/vol) yeast extract, 50 μg each of L-histidine, L-threonine, and L-tryptophan per ml, and 5 μM CCCP at pH 7.0. The photograph was taken after incubation at 30°C for 36 h.

medium supplemented with oleic acid, wild-type levels of unsaturated fatty acid were restored, with a concomitant loss of CCCP resistance of growth. The underlying biochemical alteration in the mutant strain is probably the fatty acid desaturase, which has been found to be deficient in C8 (E. A. Dunkley, Jr., unpublished data).

A variety of types of activation of membrane-associated catalysts depend upon membrane lipid composition, and the H^+/ATP stoichiometry of the ATPase has also been suggested to depend upon the membrane lipids (H. S. Van Walraven, Abstr. 1989 EMBO Workshop, Corfu). Such effects could not account for the respiration-specific nature of the increase in $\Delta G_p/\Delta\bar{\mu}_{H^+}$ at submaximal driving force that is found in C8. Some possible mechanisms whereby a change in membrane lipid fatty acid unsaturation might lead to the observed phenotype are discussed below. It should be noted here, however, that since the protonophore-resistant mutants are membrane lipid mutants, care is required to obtain consistent results with respect to the actual degree of resistance. The membrane lipid composition of bacteria is affected by a variety of nutritional and physical factors, and thus some variability in base-line protonophore resistance is readily understood, even apart from problems such as binding of specific uncouplers by sulfhydryl groups in undefined media.

Mutants of *B. subtilis*

After our initial studies of the C8 strain, we turned to *B. subtilis* because of its greater genetic accessibility and the greater ease of purification of its ATPase. Three mutant strains were isolated (strains AG1A3, AG2A, and AG3A) which grew on malate-containing medium in the presence of 5 μM CCCP (27) (Fig. 1). Importantly, although this level of resistance is well below that seen in gram-negative bacteria that contain an outer membrane (see below), there is clearly a range of uncoupler concentrations at which the wild type does not grow at all whereas the mutant strains exhibit near-normal rates of growth. All three mutant strains were also resistant to DNP. AG2A was more sensitive than the wild type to gramicidin, but less sensitive to neomycin or tributyl tin. Growth of the other two mutant strains showed the same sensitivity to these agents as the wild type.

As in C8, there were several bioenergetic differences between the uncoupler-resistant mutants of *B. subtilis* and their parent strain, although not all of those differences were quantitatively or qualitatively parallel to the differences in the *B. megaterium* pair. The respiratory rates of AG1A3, AG2A, and AG3A were slightly elevated (about 15%) over that of the wild type, although no obvious changes in cytochrome patterns were observed in reduced versus oxidized difference spectra of the membranes (27). In contrast, the ATPase activity in the membranes of the *B. subtilis* mutants was considerably higher (about 75% higher) than that of the wild type. Importantly, and again as in C8, the uncouplers were neither inactivated nor excluded. CCCP affected the level of the $\Delta\bar{\mu}_{H^+}$ identically in the wild type and in the mutants, and the passive proton permeabilities of the mutant membranes were also unchanged.

ATP synthesis was studied in detail as a function of the mode of energization and with respect to titration of the $\Delta\bar{\mu}_{H^+}$ by different uncoupling agents. Agents and conditions were chosen so that the species that had to enter and function within the membrane, e.g., protonated versus unprotonated CCCP, valinomycin with or without K^+, and nigericin carrying either a proton or potassium ion, would not have the same net charge in each instance. If the mutants were to show greater ATP synthesis at submaximal driving forces attained by each of these agents, it would eliminate as the crucial factor an alteration in surface charge or potential affecting the entry or mobility of agents with a particular charge. The mutants had unaltered sensitivity to CCCP- or nigericin-induced reduction of the $\Delta\bar{\mu}_{H^+}$. Interestingly, the mutants had greater sensitivity than the wild type to valinomycin (plus K^+)-induced reduction of the $\Delta\psi$; this observation will be discussed in connection with models of energy transduction in these mutants. In addition, one set of determinations was conducted under conditions (pH 6.2) in which both a ΔpH and $\Delta\psi$ existed, whereas the others were conducted under conditions (pH 7.5) in which the $\Delta\psi$ was the sole component of the $\Delta\bar{\mu}_{H^+}$. Maximal levels of the $\Delta\bar{\mu}_{H^+}$ were the same in the mutants as in the wild type; all the strains exhibited somewhat higher $\Delta\bar{\mu}_{H^+}$ at pH 6.2 than at pH 7.5 (Table 2). Importantly, at these comparable maximal $\Delta\bar{\mu}_{H^+}$ values, the mutants synthesized the same amount of ATP as did the wild type when energized by malate addition after starvation. Although not shown in Table 2, the $\Delta G_p/\Delta\bar{\mu}_{H^+}$ ratios for the mutants and wild type under these conditions were all close to 3.0. By contrast, at a given submaximal value of the $\Delta\bar{\mu}_{H^+}$, the mutants synthesized more ATP upon reenergization than the wild type did, regardless of the pH or the agent used to prevent maximal $\Delta\bar{\mu}_{H^+}$ generation; even in the presence of valinomycin, to which the mutants were more sensitive, these strains synthesized more ATP than the wild type did at a given $\Delta\psi$ level. In the presence of 2 μM CCCP, the $\Delta G_p/\Delta\bar{\mu}_{H^+}$ for the wild type was approximately 4.3 and that for the mutants was approximately 5.3 (27). When comparable cells were energized by a valinomycin-mediated potassium diffusion potential or by an imposed pH gradient, synthesis in both the wild type and the mutants was much lower than observed upon malate or ascorbate-PMS addition and was inhibited equally in the wild type and mutants by CCCP (A. A. Guffanti, unpublished data).

As was the case for C8, revertants of the *B. subtilis* mutants arose with a frequency consistent with a single mutation and showed recovery of the full panoply of wild-type properties (27). Again, the single underlying mutation appears to reside in the fatty acid desaturase system, result-

TABLE 2. More ATP is made by uncoupler-resistant mutants than by wild-type *B. subtilis* at submaximal $\Delta\bar{\mu}_{H^+}$ levels, and restoration of unsaturated fatty acids reverses this phenotype[a]

Expt	Wild type		AG2A		AG3A	
	$\Delta\bar{\mu}_{H^+}$ (mV)	ATP synthesis (mM)	$\Delta\bar{\mu}_{H^+}$ (mV)	ATP synthesis (mM)	$\Delta\bar{\mu}_{H^+}$ (mV)	ATP synthesis (mM)
A						
Control	−162	4.0	−157	3.9	−168	4.0
CCCP (2 μM)	−87	0.6	−82	2.5	−80	1.8
Control[b]	−191	4.7	−227	4.8	−195	4.7
Nigericin[b] (15 nM)	−104	0.1	−105	0.9	−101	2.4
Control	−160	3.7	−161	3.4	−161	3.8
Valinomycin (0.05–0.1 μM)	−102	0.8	−102	2.0	−102	1.5
B						
Control	−160	3.1	−158	2.9	−159	2.9
CCCP (2 μM)	−81	0.1	−85	1.2	−83	0.9
Grown with palmitic acid (10 μM)	−162	2.1	−160	2.7	−160	3.3
As above, assayed with CCCP (2 μM)	−85	0.2	−83	1.0	−84	1.1
Grown with palmitoleic acid (10 μM)	−155	3.1	−163	3.2	−162	2.9
As above, assayed with CCCP (2 μM)	−83	0.1	−80	0.6	−80	0.2

[a] The results presented in experiment A were compiled from reference 27, and results in experiment B were compiled from reference 57. In experiment A, cells were washed with 50 mM potassium phosphate (pH 7.5); in experiment B, cells were washed with the phosphate buffer plus 1% (wt/vol) bovine serum albumin.
[b] pH 6.2 (other experiments were performed at pH 7.5, at which there is no ΔpH).

ing in changes in the membrane lipids. We do not yet understand why the mutants exhibit slight differences in phenotype, but this might result from lesions in different components of the desaturase system. The overall activity of fatty acid desaturase of the mutants is greatly reduced, and temperature-sensitive revertants of two of the mutants possess temperature-sensitive desaturase activity (Dunkley, unpublished). The deficiency results in a reduction in the unsaturated fatty acid content from about 16% of total membrane fatty acid (all $C_{16:1}$) in the wild type to 6 to 7% in the mutants (57). There is also a secondary change in the branching pattern of the branched-chain fatty acids of the mutants such that the ratio of iso-branched chains to anteiso-branched chains increases (57). When the mutant strains were grown on medium containing palmitoleic acid, the unsaturated fatty acid was preferentially incorporated into the phosphatidylethanolamine and cardiolipin components. This incorporation restored the saturated/unsaturated fatty acid ratio of the total phospholipid to the wild-type level and restored the wild-type pattern of branched-chain fatty acids. Most importantly, these changes were accompanied by loss of resistance of growth of the mutant strains to CCCP. The synthesis of ATP upon addition of malate to starved cells was similarly less resistant to CCCP when the mutants were grown with an unsaturated fatty acid supplement (Table 2). The exogenous fatty acids were removed (by treatments that may have slightly enhanced the inherent CCCP sensitivity of all strains) after growth, so that the effects measured were due to the membrane composition, not to the assay conditions. Studies with duramycin-resistant derivatives of both the wild-type and mutant strains indicated that protonophore resistance was retained even upon mutational reduction in phosphatidylethanolamine and cardiolipin (76) and that the effect of exogenous palmitoleic acid was also demonstrable in such strains (19).

Thus far, the *Bacillus* mutants described above are the only uncoupler-resistant bacteria that clearly do not exclude or inactivate the uncoupler. It is of great interest that they are resistant only to modest concentrations of uncouplers and have all proved to be mutants with altered saturated/unsaturated fatty acid ratios in their membrane lipids. When uncoupler-resistant strains of *B. subtilis* were challenged with higher CCCP levels, resistant strains were isolated that had elaborated a slime layer which excluded the protonophore, as was also found for stationary-phase cells of *B. subtilis* grown on some media (27). This should be noted in connection with mutants of *E. coli* that are described below, in which the outer membrane properties appear to be central. It will also be interesting to examine recent isolates of *Bacillus firmus* OF4 that are resistant to low concentrations of CCCP (P. G. Quirk, unpublished data), since this organism has few or no unsaturated fatty acids in its membrane (11).

How Might the Membrane Lipid Changes of Protonophore-Resistant *Bacillus* Species Result in the Bioenergetic Phenotype?

The data on protonophore-resistant mutant strains of both *B. megaterium* and *B. subtilis* support the conclusion that a single mutation in the fatty acid desaturase system results in an increase in the saturated/unsaturated fatty acid ratio of the membrane phospholipids. All of the resulting phenotypic changes can be explained in terms of a parallel coupling model (such as proposed by others; see, e.g., references 21 and 91) in which proton flow between pumps and sinks may occur by a chemiosmotic bulk-to-bulk route and, in parallel, by some more direct route in which complete equilibration of the protons with the external bulk phase does not occur. In connection with the uncoupler-resistant *Bacillus* mutants, we hypothesize that the change in the membrane lipids leads to a higher resistance to proton flow through the ATPase to and from the external bulk phase and to an enhanced ability of the ATPase to capture protons from the more direct, localized pathway from the respiratory chain. Thus, the ratio of pathway utilization is shifted in the mutants toward the localized mode and away from the bulk mode. Such a change in the relative use of coupling modes could account for the phenotypic characteristics in the following ways. First, the superior ATP synthesis in the mutants at submaximal levels of the bulk $\Delta\bar{\mu}_{H^+}$ would be explained by their greater utilization of protons that are not measured as part of the bulk force. If another process such as ion-coupled solute transport were not able to utilize such a localized mode of

coupling, it would be fully sensitive to uncouplers, and, indeed, glycine transport and glutamate transport by C8 were found by Decker and Lang (16) to be CCCP sensitive. Effective and rapid movement of protons through localized pathways might account for the increased respiratory rates of the mutants in the absence of any obvious change in respiratory-chain components. By contrast, at least some of the mutants show less effective utilization of artificial gradients, because the resistance of the bulk pathway is elevated relative to that in the wild type. Consistent with such a proposed decrease in bulk-to-bulk proton movement through the ATPase is the enhanced valinomycin sensitivity exhibited by the uncoupler-resistant mutants of *B. subtilis*. As increasing concentrations of valinomycin (in the presence of external K^+) titrate the $\Delta\psi$, the wild type may pump protons out into the bulk more effectively than the mutants. Hence, the concentration of valinomycin needed to reduce the $\Delta\psi$ to some given level is higher in the wild type than in the mutants. The concomitant increase in resistance of the bulk pathway with the enhanced use of localized coupling would be crucial. Such a high resistance would act as a kinetic barrier for protons entering the ATPase, since at low bulk $\Delta\bar{\mu}_{H^+}$ protons from localized pathways would otherwise flow out into the external phase rather than inward to catalyze ATP synthesis. The continued presence of some bulk $\Delta\bar{\mu}_{H^+}$ would presumably be necessary for the same reason. We have not observed ATP synthesis by uncoupler-resistant mutants at $\Delta\bar{\mu}_{H^+}$ levels much below -50 mV.

How could the membrane changes lead to such a shift in relative utilization of proton pathways even if parallel coupling existed? There are a variety of ways in which this might occur. Although they are completely speculative at this time, we will present them briefly because they may stimulate direct experimental examination of these kinds of models. First, the increase in the ratio of saturated/unsaturated fatty acids of the membrane phospholipids might increase the relative proportion of bulk membrane lipid in the gel phase rather than in the liquid-crystalline phase at physiological temperatures. Integral membrane proteins might then be relatively excluded from the larger patches of gel phase membrane, and, consequently, their concentration in the liquid-crystalline phase would then be higher than their overall concentration in the wild-type membrane. In one model of localized proton flow, direct proton movement depends simply upon the frequency of physical collision of the proton pumps and the proton sinks in the membrane. A high frequency of collision could allow a proton to be handed off directly, perhaps between proton-binding residues within the membrane, before it has a chance to follow the entire route out into the bulk. We have suggested this mode of localized transfer in connection with alkaliphilic bacteria, whose bioenergetic dilemma has much in common with that of uncoupler-resistant *Bacillus* species (59). Slater (105) had earlier presented a more general formulation in which collisions were a critical element. The increased concentration of pumps and sinks in the reduced area of liquid-crystalline-phase membrane of uncoupler-resistant mutants might increase the frequency of productive, proton-transferring collisions and enhance localized flow. The patterns of particle (presumably protein) distribution visualized in freeze-fracture electron micrographs of *E. coli* plasma membranes by Ingram et al. (43) show an enormous difference between control and palmitate-grown cells (Fig. 2). It should be noted, however, that the interpretation of such observations may be complicated (12); i.e., they might not reflect a phenomenon that occurs in vivo. The presence of branched-chain fatty acids in *Bacillus* species would probably prevent dramatic lateral phase separation of this kind in any event (8, 29, 64). Nonetheless, a much more modest localized increase in the pump and sink concentration by this kind of mechanism, without any profound separation of phases, might produce the level of resistance observed in the *Bacillus* mutants. It should be noted here that cells of both wild-type and uncoupler-resistant mutants of *B. megaterium* and *B. subtilis* exhibit an increased resistance of growth to CCCP at elevated growth temperatures (10, 57). Unfortunately, that type of experiment does not distinguish between the effects of growth temperature on membrane lipid composition and the possible effects on energy transduction that could arise from increased collision frequencies between pumps and sinks at higher temperatures. We have speculated that the enhanced resistance might be attributable directly to reduced synthesis of unsaturated fatty acid at high temperature (10, 57); however, it is quite possible that partial escape from the protonophoric effects of CCCP is facilitated by enhanced mobility of the proteins within the membrane, independent of compositional changes.

In addition to the formation of dynamic domains that increase local concentrations of pumps and sinks, there are other ways in which the lipid changes could lead to enhanced localized or incompletely delocalized energy transduction. For example, formation of some kind of stable domain, in which the proton pumps and the ATP synthase interact, could be enhanced by the increase in the ratio of saturated to unsaturated fatty acids in the membrane lipids. Rottenberg (91) has proposed that such domains might facilitate parallel energy coupling. Lipid changes might also alter the ability of the phospholipid headgroups to form proton-transporting acid-anion dimers of the kind proposed by Haines (30). In connection with this proposal, though, the retention of protonophore resistance by duramycin-resistant second mutants with extremely low phosphatidylethanolamine and cardiolipin levels (19) would require that some other phospholipid formed the acid-anion dimers. It may be possible to distinguish between explanations of the lipid effects by performing cross-linking experiments, by performing experiments in which the distance between pumps and sinks is manipulated by fusion of vesicles with liposomes of various compositions, and/or by establishing a reconstituted system containing the ATP synthase and a proton pump at various concentrations in various lipids.

A different kind of effect of the membrane lipid change that could confer protonophore resistance would be a protection of locally transferred protons from the decoupling effects of protonophores. As described below, Rottenberg (91) has proposed that whereas some uncouplers, especially ionophores such as valinomycin, function entirely by collapsing the bulk electrochemical gradient, others, such as the weak-acid protonophores, both collapse the transmembrane gradient and exhibit decoupling effects. Decoupling involves the disruption of the putative localized proton pathway, e.g., by the ability of the anionic intramembrane form of the protonophore to pull a proton away from an intramembrane site. More highly saturated membrane lipids might, then, protect the sensitive protonated residues in the mutants. This mechanism would not fully account for the phenotype, however, since the mutants also synthesized more ATP than their parent strain when the $\Delta\bar{\mu}_{H^+}$ was lowered by using valinomycin plus K^+.

In addition to enhancing localized coupling and/or protecting the localized pathway from the decoupling effects of the protonophore, the lipid change may also decrease proton

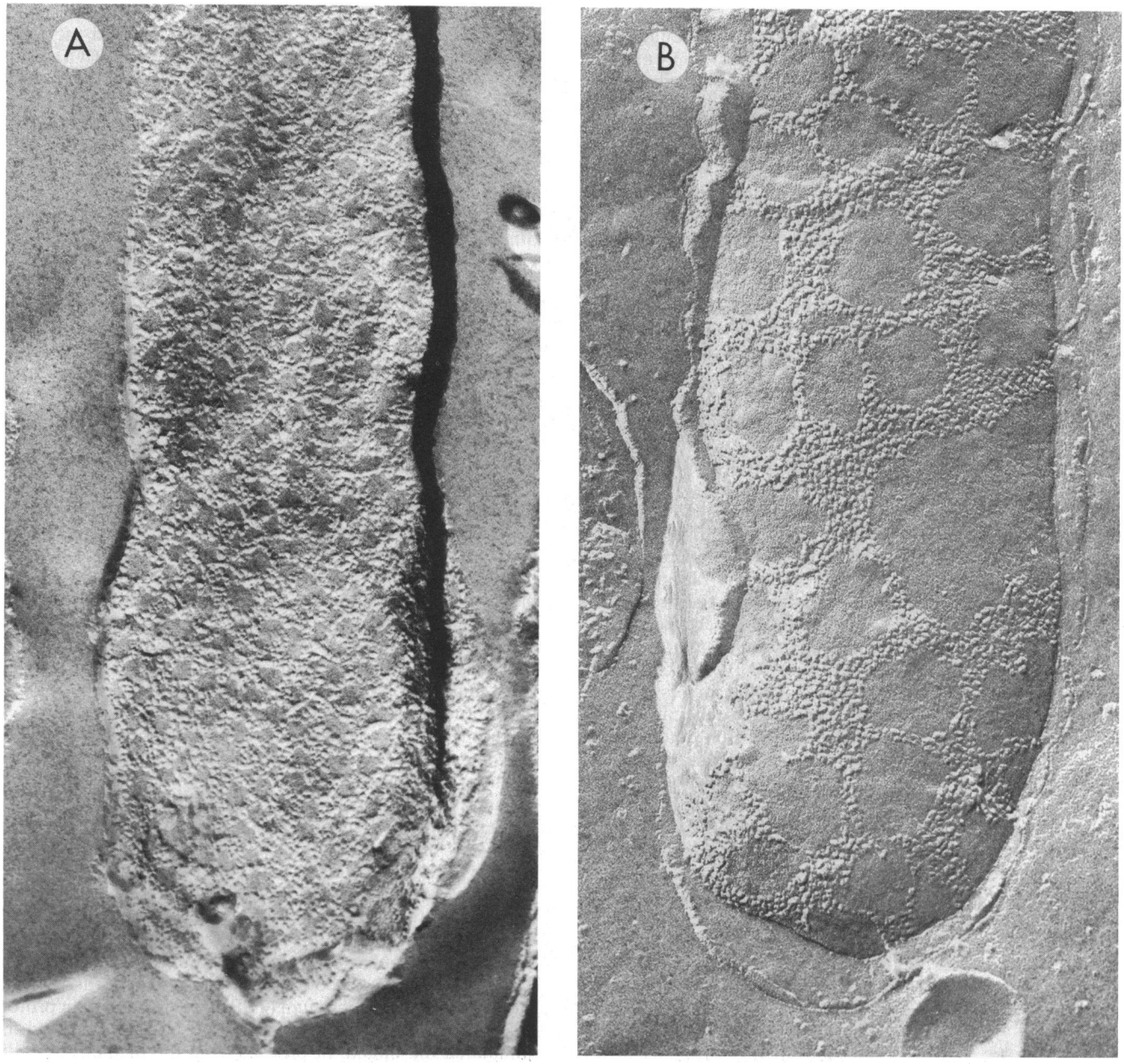

FIG. 2. Freeze-fracture electron-microscopic images of membranes from *E. coli* cells that had been grown with or without palmitate in the medium. These micrographs were graciously provided by L. O. Ingram and G. W. Erdos. (A) Preparation from *E. coli* grown at 37°C without palmitate; (B) preparation from comparable cells grown at 37°C with palmitate; both samples were held at 37°C before quenching. Final magnification, ×83,200. Part of these images were published as half of Fig. 4 in reference 43.

loss to or entry from the bulk (e.g., as evidenced by the valinomycin sensitivity of the *B. subtilis* mutants and the lesser efficacy of imposed artificial gradients in general and in at least one of the mutants relative to its wild type). It will be of interest to reconstitute the wild-type F_0 into proteoliposomes prepared from wild-type versus mutant lipids and test whether the lipids influence the proton conductivity of the F_0.

Finally, it should be noted that were conventional, nonmarine aerobes to possess a chemiosmotic Na^+ cycle, in which a $\Delta\bar{\mu}_{Na^+}$ replaces $\Delta\bar{\mu}_{H^+}$ for bioenergetic work, protonophore resistance in such organisms could be accounted for by a switching over to the use of an Na^+ cycle. There would have to be an inherent capacity for Na^+-coupled ATP synthesis and a primary (probably respiration-coupled) Na^+ pump. Skulachev, who has promoted the notion of a widespread use of such an Na^+ cycle (102), has indicated that its presence might underlie the observations with protonophore-resistant bacteria (4, 103). Although conceptually appealing, this proposal is a rather large leap from the base of existing data. An analogous cycle is elegantly established in anaerobic, salt-requiring *Propionigenium modestum* (18), but there is no comparable evidence for an Na^+ cycle that, alone, allows normal growth of an aerobic nonmarine organism by using oxidative phosphorylation. All the other, nonmarine, bacteria that exhibit primary substrate-coupled Na^+ pumping do not possess an Na^+-coupled ATPase (18). Even in the marine vibrios that have both elements of a cycle, i.e.,

a primary, respiration-coupled Na^+ pump and an ATPase that may partially couple ATP synthesis to Na^+ under some conditions, the Na^+ cycle apparently cannot support normal growth on nonfermentable carbon sources in the absence of a chemiosmotic H^+ cycle (95). Less has been demonstrated in terms of the properties and possible role(s) of a putative primary, respiration-coupled Na^+ pump in marine *Bacillus* strain FTU (101). The recent suggestion (4) that a conventional bacterium, i.e., *E. coli*, may induce a primary respiration-coupled Na^+ pump when grown at pH values at which the $\Delta\bar{\mu}_{H^+}$ is low, needs confirmation by more conclusive ion flux experiments, and, finally, by biochemical characterization of the putative pump. As will be discussed below, that suggestion is, in any event, not immediately relevant to the protonophore-resistant strains of *E. coli* that have been isolated to date. Could a similar phenomenon account for the protonophore-resistant phenotype of the *Bacillus* mutants described here? No. CCCP, nigericin, and valinomycin (plus K^+) all reduce the $\Delta\bar{\mu}_{H^+}$ components (in many experiments composed only of a $\Delta\psi$) at least as well in the uncoupler-resistant *Bacillus* mutants as in the wild type under the conditions in which ATP synthesis is resistant. Use of an Na^+ cycle would result in a form of resistance in which the uncoupler failed to lower the $\Delta\psi$ as much in the mutant because it failed to allow sufficient ion flux to counteract the Na^+ pump. In addition, and apart from the fact that these mutants are single, membrane lipid mutants, their resistance does not depend upon the presence of Na^+, and neither the wild-type nor the protonophore-resistant mutant cells exhibit Na^+-coupled ATPase activity (D. B. Hicks, unpublished data). In the diffusion potential experiments in which the mutants functioned poorly, large electrochemical gradients of Na^+ were present (since the diffusion potential was achieved by dilution of valinomycin-treated, potassium-replete cells into low K^+, high Na^+ buffer). This eliminates the involvement of an Na^+-coupled synthase in the biology of these mutants. Furthermore, it should be possible to document the ion selectivity of the F_0 of mutant versus wild-type strains reconstituted into mutant versus wild-type lipids. Such a reconstituted system will also be of great interest as a model system that may allow tests of several of the above hypotheses, since the protein-to-lipid ratios, the lipid composition, and the temperature will be controllable.

MUTANTS OF *E. COLI*

Description of the Mutants Reported

The first report of uncoupler resistance in *E. coli* appeared in 1980, when Date et al. isolated a mutant with a spontaneous mutation to CCCP resistance; this mutant was designated strain SWL14 (15). CCCP at 5 μM substantially inhibited proline transport in the parent strain, but had little effect on the mutant. Surprisingly, proline transport appeared more resistant in vesicles than in whole cells, despite the intact outer membrane of the latter; the reason for this is unclear. The conversion of M13 bacteriophage procoat protein to mature coat protein also became resistant to CCCP in the mutant. As this conversion apparently required a $\Delta\bar{\mu}_{H^+}$, the latter may have been retained in strain SWL14, despite the presence of uncoupler.

A whole series of uncoupler-resistant *E. coli* strains has been isolated by Ito and co-workers. Derivatives of strain KH434 were originally selected for resistance to tributyl tin (44). This reagent may either inhibit the ATP synthase directly (50) or catalyze transmembrane Cl^-/OH^- exchange (100); however, Ito and Ohnishi (44) used medium lacking added Cl^-. All the 21 mutants isolated showed increased resistance to one or more of: azide (14 of 21); DCCD (10 of 21), CCCP (10 of 21), and PCP (18 of 21). Two of the mutants were more resistant to all four compounds, and one of these, designated strain SM434, behaved as a temperature-dependent uncoupled mutant, able to grow on succinate at 30°C but not at 42°C. Introduction of an F′ factor containing the wild-type 80- to 89-min chromosomal region restored the ability of SM434 to grow on succinate at 42°C, but DNA other than that of the *atp* operon may have been responsible. Of 20 spontaneous revertants to Atp^+, only 3 reverted to wild-type sensitivities to the five agents, whereas the other 17 retained resistance to some or all of them, indicating that the Atp^+ phenotype could be restored by secondary mutations. Two of the mutant alleles cotransduced with *ilvA* (85 min) and were believed to reside in the *atp* operon (84 min).

A further series of seven isolates was selected for resistance to PCP. All were additionally resistant to tributyl tin, four to CCCP, three to azide, and one to DCCD (45). CCCP stimulated respiration and induced proton influx in cells of the parent strain, but not in the three mutants examined or in vesicles of one of these, strain CM22 (46). Unfortunately, the magnitude of the initial ΔpH was not determined, leaving open the possibilities that CM22 vesicles were already leaky to protons or had unusual $\Delta\bar{\mu}_{H^+}$ patterns before the uncoupler was added. However, it is most likely that CCCP was simply ineffective in the mutant cells and vesicles. Strain CM22 exhibited a temperature-sensitive uncoupled phenotype and a reversion frequency of 10^{-8}, suggesting a single point mutation. All three mutations cotransduced with *ilvA*, but no further mapping data have appeared.

Another uncoupler-resistant strain of *E. coli*, designated UV6, has been described by Bragg and colleagues. Growth of strain UV6 was resistant to CCCP (250 μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and tributyl tin (in medium lacking Cl^-), but not to DNP or other inhibitors of protein synthesis and of the ATPase (99). Uptake of proline by intact cells was resistant to CCCP (see also reference 47) and was only weakly inhibited by tributyl tin, even in the presence of 40 mM Cl^-. The ΔpH of UV6 was much less susceptible to collapse by CCCP; unfortunately, parallel measurements of membrane potential could not be made because both parent and mutant cells took up the tetraphenylphosphonium (TPP^+) probe poorly. In proton pulse experiments, by contrast, both CCCP and tributyl tin stimulated the uptake of protons from the medium by both UV6 and its parent. In these experiments, protons may have equilibrated only across the outer membrane. Interestingly, succinate-grown UV6 maintained an intracellular phosphate concentration of 75 mM, reducing ΔG_p from 45 kJ/mol (parent strain) to 39 kJ/mol. Growth of strain UV6 in the presence of uncoupler resulted in an elevated protein/lipid ratio, causing a decrease in overall membrane fluidity, even though the remaining phospholipids contained a higher proportion of unsaturated side chains (37). Under these conditions, CCCP also caused the incorporation of elongation factor Tu into the outer membrane (99).

E. coli is, in general, more resistant to uncouplers than *Bacillus* species are. For example, the parent strain used by Ito and Ohnishi (44) was resistant to 25 μM CCCP, whereas even uncoupler-resistant strains of gram-positive *Bacillus* species generally tolerate no more than 5 μM CCCP in aerated liquid cultures (26, 27). Interestingly, considerably more uncoupler was required to inhibit growth of and amino acid uptake in *E. coli* than for several *Vibrio* species (66),

even though the latter species may have a chemiosmotic Na^+ cycle that could reduce their overall protonophore sensitivity (102, 103). Presumably, resistance to uncouplers depends heavily on the properties of the outer cell layers. We should also note the recent suggestion that primary, respiration-coupled Na^+ pumping may occur in *E. coli* grown at low $\Delta\bar{\mu}_{H^+}$ (4), as discussed in an earlier section. If this suggestion is confirmed by further determinations, it will be especially important to examine putative protonophore resistance at pH values at which the chemiosmotic proton cycle is required for bioenergetic work or under conditions in which there is cross-resistance to agents that would abolish the $\Delta\bar{\mu}_{Na^+}$ as well as the $\Delta\bar{\mu}_{H^+}$. It is clear from the work of Bragg and colleagues that the outer membrane of *E. coli* may pose a considerable barrier to uncouplers, even in "nonresistant" strains. Sedgwick et al. (99) noted that an *E. coli ompA* strain lacking outer membrane protein 3a showed heightened sensitivity to CCCP. Cell wall properties may also account, in part, for the relative insensitivity of the archaebacterium *Methanobacterium thermoautotrophicum* to hydrophobic, lipophilic uncouplers (49); DNP was much more effective.

Such considerations led Beechey and colleagues (47, 86) to study uncoupler-resistant mutants of *E. coli* Doc-S, a strain whose defective outer membrane rendered it unusually sensitive to deoxycholate (1) and to uncouplers. Mutant strain TUV was selected for resistance to 4,5,6,7-tetrachloro-2-(trifluoromethyl)benzimidazole (TTFB), and a second strain, CUV, was derived from TUV. Strain TUV was cross-resistant to CCCP, FCCP, and triphenyl tin, but not to DNP (99), whereas strain CUV displayed a further enhanced resistance to FCCP and CCCP (but not to TTFB or triphenyl tin) and some resistance to DNP (86). Both resistant strains remained highly sensitive to deoxycholate (47), and it was inferred that uncouplers would also have equal access to the energy-transducing membrane of the mutants. This was demonstrated directly with starved cells of strains Doc-S and TUV by using phosphorus nuclear magnetic resonance spectroscopy; TTFB collapsed the ΔpH at an identical rate in both strains and caused the rapid depletion of intracellular ATP (86). When both strains were supplied with respiratory substrate (succinate or glycerol), however, strain TUV showed superior pH homeostasis and retained substantial amounts of ATP when challenged with uncoupler. Other lines of evidence suggested that the mutational alteration was, nevertheless, in the permeability of the outer membrane. The membrane potential of strain Doc-S could be readily determined by using TPP^+, but strains TUV and CUV failed to take up the probe unless pretreated with EDTA (see also reference 99). In addition, strains TUV and CUV were much more resistant than their parent to novobiocin, an inhibitor of DNA gyrase. Such resistance is most likely to arise from a reduced permeability to the agent. Preliminary mapping data suggested that strain TUV carried a mutation in the *rfa* operon, a group of genes concerned with outer membrane lipopolysaccharide synthesis (78).

Does a Coherent Picture Emerge from These Observations?

Under certain conditions, *E. coli* can grow in the presence of an uncoupler and in the apparent absence of a $\Delta\bar{\mu}_{H^+}$ (56, 75). A fermentable carbon source and an extracellular pH close to 7.5 are generally required. High (>200 mM) Na^+ or K^+ concentrations sensitize strains to the uncoupler (24, 66). Importantly, the investigations reviewed above have used conditions under which a $\Delta\bar{\mu}_{H^+}$ was thought to be mandatory for growth. In particular, a near-neutral growth pH with the provision of only a nonfermentable carbon source (usually succinate or glycerol) necessitated ATP synthesis by oxidative phosphorylation, and active transport was required for the accumulation of succinate and/or amino acids. Thus, the uncoupler-resistant strains either maintain their $\Delta\bar{\mu}_{H^+}$ in the presence of uncoupler or can perform bioenergetic functions at submaximal levels of $\Delta\bar{\mu}_{H^+}$; the former appears to be the basis for the mutations described thus far in *E. coli*.

Uncoupler-resistant mutants of *E. coli* show pleiotropic phenotypic changes; this is particularly apparent in the best-characterized strains, UV6 (98, 99) and TUV (47, 86). Energized cells of both strains exhibit many similarities: multiple cross-resistance to membrane-active agents but retention of sensitivity to DNP; uncoupler-resistant active transport in whole cells; enhanced maintenance of a ΔpH in the presence of uncouplers; and impaired uptake of TPP^+. Analysis of strain UV6 was complicated by the apparent uncoupler effects involving the outer membrane (99). The use of nuclear magnetic resonance spectroscopy (86) obviated some of the possible effects of this kind with strain TUV, since the small periplasmic volume could not contribute significantly to the spectra. In general, however, there have been few measurements of relevant energetic parameters. The ΔpH component of $\Delta\bar{\mu}_{H^+}$ appears to be resistant to dissipation in all cases when nonstarved cells were used (45, 46, 86, 99). As noted above, TTFB collapsed the ΔpH of starved cells of strain TUV and its nonresistant parent at identical rates (86). Although the collapse was slow, possibly a consequence of the very concentrated cell suspensions used, this is perhaps the clearest evidence for an unimpaired permeation of uncoupler in any uncoupler-resistant strain of *E. coli*. Even in this strain, however, unstarved cells appeared to exclude the uncoupler, as discussed above.

Quirk et al. rationalized their apparently contradictory permeability data by postulating a reversible outer membrane change, with uncoupler penetrating when the cell membrane was deenergized but being excluded by energized cells (86). A precedent for such a phenomenon has been provided by fluorescence studies; the probe *N*-phenyl-1-naphthylamine was excluded from the inner membrane of *E. coli* unless the cells were treated with FCCP (36) or other deenergizing reagents (77). Interestingly, the TonB cell membrane protein contains two highly charged sequences which could respond to membrane energization by interacting with the outer membrane and mediating transport processes across it (39). Thus, the properties of the uncoupler-resistant strain TUV may be of some bioenergetic interest, although not of the type originally envisaged (47).

The similarities between strains TUV and UV6 suggest that there may be a related underlying mechanism for their resistance; although Sedgwick and Bragg (99) failed to detect any outer membrane changes in UV6, such an alteration could be subtle. For example, strain TUV retains an abnormally high permeability to deoxycholate (47). The retention by both strains of sensitivity to the relatively hydrophilic DNP could arise after this compound gained access to the cell membrane through the (unaltered) pores in the outer membrane. In addition, the proximity of the *rfa* (81 min) and *atp* (84 min) operons makes it possible that some of the mutations initially assigned to the latter (44–46) actually reside in the former. A temperature-sensitive uncoupled phenotype would, however, be difficult to explain in this way. Further genetic analysis of these strains would be valuable.

To date, no equivalent to the uncoupler-resistant *Bacillus*

species has been described for *E. coli*. Selection of such a mutant would pose some problems. Although it is relatively easy to detect an increase in the CCCP resistance of *B. subtilis* from 1 to 5 μM, the equivalent phenotype in *E. coli* might well resist 25 μM instead of 20 μM and would have to be separated from other mutants resisting more than 100 μM CCCP. Moreover, the dependence of *E. coli* on a $\Delta\bar{\mu}_{H^+}$ under the conditions used for selection would have to be confirmed by eliminating the remote possibility, for example, that all the requisite solute symporters and the ATP synthase could use Na^+ under those conditions. Nevertheless, such a project would be well worth attempting; the chances of success might be enhanced by starting with an outer-membrane mutant (preferably resulting from deletion of a well-characterized gene) and using a more potent uncoupler such as FCCP.

RELATED OBSERVATIONS AND PHENOMENA

Uncoupler-Binding Proteins and Other Uncoupler Effects

As noted in the Introduction, one of the successes of Mitchell's chemiosmotic theory has been its ability to explain the action of uncoupling agents (69). However, although many excellent correlations between protonophoric activity in liposomes and uncoupling activity in mitochondria and chloroplasts have been documented (6, 67, 70–73), there are also observations suggesting that some agents have energy-coupling effects other than simple dissipation of bulk gradients. For example, there are uncoupling effects in both mitochondria (65) and chloroplasts (83, 97) that are incompletely explained by the increase in bulk-to-bulk proton permeability caused by the agent. Rottenberg (91) has introduced the term "decoupling" to describe the effects of agents such as anesthetics, fatty acids, and even gramicidin (under some specific conditions) on oxidative phosphorylation or photophosphorylation. The underlying observations are an uncoupling of phosphorylation from primary proton pumping without an equivalent decrease in the $\Delta\bar{\mu}_{H^+}$ that is the putative intermediate, or sometimes with almost no decrease therein (84, 90, 92, 94). As mentioned above, Rottenberg proposes that decoupling occurs through effects on a direct intramembrane coupling pathway, with the agent transporting protons or facilitating leaks from within such pathways to the bulk. Rottenberg and co-workers (84, 91) have distinguished three types of agents: (i) those, such as fatty acids and gramicidin (under certain conditions, including very low alkali cation concentrations), whose decoupling effects are much more pronounced than their chemiosmotic uncoupling effects; (ii) those such as the weak-acid protonophores, that exhibit a mixture of chemiosmotic and decoupling effects; and (iii) those, such as nigericin and valinomycin, that function chemiosmotically. Although other effects have been ascribed to some of the decoupling agents (2, 65, 85), the model of decoupling described above is supported by various observations that include precedents in which a proton is indeed abstracted from intramembrane sites. For example, the azide-stimulated deprotonation of the Schiff base in halorhodopsin has been described in such terms by Lanyi (62). The relative sensitivities of oxidative phosphorylation by various uncoupler-resistant *Bacillus* mutant strains and their parent strains to different categories of uncoupling agents might be illuminating with respect to the details of the mechanism of resistance. A mutant that relies more heavily on localized pathways of proton movement than the parent, and hence is moderately resistant to lowering of the bulk $\Delta\bar{\mu}_{H^+}$, might be exquisitely sensitive to a decoupler.

Another proposed departure from the classical chemiosmotic model of uncoupler action is that of specific interactions of at least certain protonophores with energetically relevant proteins within the coupling membrane (55). Decker and Lang (17) and Ito and Ohnishi (44) both proposed that the uncoupler-resistant strains under investigation (*B. megaterium* and *E. coli* mutants, respectively) might have altered binding of protonophores to an ATPase-associated protein. Initial studies directed at the possibility of such an interaction revealed only linear, monophasic binding of several uncouplers to mitochondria, consistent with a simple partition effect (5, 106, 110). However, the use of radioactive uncoupler derivatives, modified by the addition of a photoaffinity-labeling azido group, allowed much more sensitive assays of both covalent and equilibrium binding to be performed and has provided some evidence for specific binding. Thus, 40% of 2-azido-4-nitrophenol was reported to bind to beef heart mitochondrial proteins of 20 to 30 kilodaltons (kDa) (33). The specific, noncovalent binding of 2-azido-4-nitrophenol (K_d = 6 μM) was inhibited competitively by other uncouplers (DNP, PCP, CCCP, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide [S13]) but not by valinomycin, antimycin, or arsenate. DNP competed less effectively under photolytic conditions. Similar binding was observed with mitochondria from rat livers, *Saccharomyces cerevisiae*, *Euglena gracilis* (31), and hamsters (14), but further studies yielded conflicting data. Kurup and Sanadi (60) reported the labeling (in bovine heart submitochondrial particles) predominantly of 9- and 20-kDa proteins and proposed the former to be the DCCD-binding subunit of the ATP synthase. Hanstein, Hatefi, and colleagues later reported that 60% of covalently bound 2-azido-4-nitrophenol was associated with proteins of 56 and 31 kDa, with minor components of 43 and 12 kDa (23, 32, 34). The 56-kDa protein was shown to be the α subunit of F_1; DNP enhances the binding of phosphate to both purified and membrane-bound F_1, possibly at a catalytic site (81, 82). From amino acid composition data, the hydrophobic 31-kDa protein has been suggested to be a dimer of the 15-kDa F_0 component (7) termed F_B by Sanadi (96).

Using 4-azido-2-nitrocarbonylocyanidephenylhydrazone (N_3CCP), a more potent and hydrophobic uncoupler than 2-azido-4-nitrophenol, Katre and Wilson (52, 53) observed highly specific binding (K_d = 0.6 μM) to a 10- to 15-kDa F_0-associated polypeptide of pigeon heart mitochondria, possibly the F_B monomer or the DCCD-binding subunit. Other uncouplers competed for binding under nonphotolytic conditions. Similar high-affinity binding sites of 10- to 15-kDa proteins were found in the procaryote *Paracoccus denitrificans* and the eucaryotic microorganism *Tetrahymena pyriformis* (54) and in blowfly mitochondria (35). Interestingly, both *P. denitrificans* and *E. coli* contain protein that is immunologically cross-reactive with F_B (48, 96). Uncoupler- and inhibitor-binding sites have also been found in rat liver submitochondrial particles labeled with N_3CCP, DCCD, or the trialkyl tin compound dibutylchloromethyl tin (80). In each case, radioactivity was concentrated in a single band of 10 to 16 kDa, but further electrophoretic and chromatographic separation indicated that three different proteins were involved. A possible approach to the role of uncoupler binding has come from recent work of Terada et al. (108) with a nonuncoupling derivative of 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile (SF6847) in which the acid-dissociable proton was replaced by a methoxy group. Meth-

oxy-SF6847 at 20 μM has little effect on ATPase activity or ATP-P_i exchange, but inhibits state 3 respiration by up to 80%. However, its mechanism of action and specificity of binding require further characterization.

In view of the suggestions of specific uncoupler interactions with membrane proteins, the possibility that an uncoupler-resistant mutant phenotype involves an alteration in such an interaction is understandably appealing. However, the evidence is, to the contrary, that those phenotypes arose from cytoplasmic membrane lipid changes or from alterations in the outer membrane. Indeed, no functional significance of the photoaffinity labeling of membrane proteins with uncoupler derivatives has yet been established. Moreover, it is notable that only some protonophores exhibit protein binding, and even among those that do, analogs that have differing efficacies as protonophoric versus protein-binding reagents uncouple mitochondrial oxidative phosphorylation in direct proportion to their protonophoric activity (3). It has also been long recognized that the concentration of protonophore needed to completely uncouple is generally much lower than that required for a stoichiometric interaction with the relevant membrane-associated protein(s) (109).

Uncoupler- and Fusion-Resistant Mutants of Eucaryotic Cells

Lancashire and Griffiths isolated *Saccharomyces cerevisiae* mutants that were resistant to various combinations of membrane-active and other antibiotic agents (61). Mutants of *S. cerevisiae* were selected for resistance to triethyl tin sulfate and were found to fall into two groups. One group exhibited cross-resistance to oligomycin, valinomycin, and at least two uncouplers, as well as erythromycin, antimycin, chloramphenicol, and other compounds. The other group was cross-resistant only to the uncoupling agent 1799 [bis (hexafluoroacetonyl)-acetone]. Subsequently, Carignani et al. (9) studied single-step mutations of *S. cerevisiae* for resistance to the mitochondrion-specific dye rhodamine 6G. Again, the pattern of cross-resistance was complex, and genetic studies suggested an interesting involvement of both nuclear and cytoplasmic factors in the determination of the phenotype. However, the bioenergetics of these mutants have not been extensively examined. More recently, Wiseman et al. (112) have isolated variants of cultured human cells (VA2-B) that are resistant to rhodamine 6G and rhodamine 123. Studies of rhodamine fluorescence led the authors to speculate that the $\Delta\psi$ across the mutant mitochondrial membrane may be altered relative to that of the wild type.

The eucaryotic cell mutant that is most bioenergetically comparable to some of the procaryotic isolates is an uncoupler-resistant line of Chinese hamster ovary cells described by Freeman et al. (22). The uncoupler-resistant clone UH_5 required four times as much S13 as its parent to inhibit respiration and nine times as much S13 to inhibit growth. UH_5 was also resistant to SF6847, CCCP, and DNP, but not to oligomycin or venturicidin. Oxidative phosphorylation by isolated mitochondria of UH_5 showed a similarly increased resistance to protonophoric uncouplers. Interestingly, the mitochondria from the mutant contained higher levels of cytochrome oxidase than did the parent, measured in reduced versus oxidized difference spectra, and lower ATPase activity. It would be of great interest to determine whether these changes might be secondary to an alteration in the membrane lipid composition of the inner mitochondrial membrane or to a change in pump concentration, e.g., leading to enhanced localized proton transfer along the lines discussed above. The lower ATPase activity, reminiscent of the C8 mutant of *B. megaterium*, could represent a different effectiveness of H^+ movements to the bulk without representing a decrease in the synthase level.

A brief mention is made here of another group of eucaryotic mutants, even though it is their membrane lipid phenotype that draws attention rather than protonophore resistance. One major element of that membrane lipid phenotype is strikingly similar to that described for protonophore-resistant *Bacillus* species, and we include it here with the thought that some common thread may emerge. Roos and Davidson (89) isolated a series of mutants of mouse fibroblast cells that were resistant to the fusion-inducing effect of polyethylene glycol. Subsequent studies by Roos and Choppin (87, 88; D. S. Roos, *in* S. Ohki, ed., *Molecular Mechanisms of Membrane Fusion*, in press) demonstrated that polyethylene glycol fusion resistance was correlated with changes in the membrane lipids and was inversely correlated with the fusibility of the cells by murine hepatitis virus. There was an increase in the amount of neutral lipid, including a major increase in an unusual ether-linked lipid, and there was also an increase in the ratio of saturated to polyunsaturated fatty acids from 1:1 in the parent line to 4:1 in resistant mutants. Moreover, growth of the parent cells in saturated fatty acids increased the resistance of these cells to polyethylene glycol-induced fusion and had a converse effect on virus fusibility; growth of the mutants in *cis*-polyunsaturated fatty acids (but, interestingly, not in *trans*-polyunsaturated fatty acids) caused them to lose their resistance to polyethylene glycol-mediated fusion.

Ethanol Tolerance

Finally, we would note that, a priori, resistance to organic solvents might be expected to pose an overlapping set of challenges with uncoupler resistance. That is, if the membrane becomes generally permeabilized, even modestly, energy transduction might be among the most vulnerable vital processes. It is outside the scope of this review to summarize an extensive literature on all sorts of solvent resistance. However, because of the similarity of some of the central issues, we will consider a few selected studies in connection with aspects of uncoupler resistance that have been discussed above.

Rottenberg, Rubin, their colleagues, and others have studied the uncoupling and fluidizing effects of ethanol on mitochondrial membranes in normal rats and in rats that were chronically fed with ethanol as part of a diet that was matched with the control diet in caloric content. Mitochondria from normal animals exhibit pronounced disordering upon acute in vitro treatment with ethanol. Such ethanol treatment increased state 4 respiration and decreased state 3 respiration, consistent with a weak uncoupling effect. This effect was much more pronounced above 35°C than at lower temperatures (93). Interestingly, mitochondria from ethanol-fed animals were more resistant to the acute disordering and uncoupling effects of ethanol and had transition temperatures for respiration that suggested lipid modifications (93). Ethanol decreased the order parameter, measured with the spin probe 5-doxylstearic acid, in mitochondria from normal animals but not from those that had been chronically fed with ethanol. Comparable effects were observed in experiments in which mitochondrial lipid vesicles were used. Studies of phospholipid composition revealed a number of

changes, the most dramatic being that the cardiolipin from the mitochondria of ethanol-fed animals had a higher saturated/unsaturated fatty acid ratio (111). Cunningham and Spach (13) suggest that there is depressed reacylation of phospholipids in mitochondria from ethanol-fed animals and found longer chains and increased saturation of the fatty acids. Notably, in studies by Ellingson et al. of lipid vesicles containing various combinations of mitochondrial phospholipids from the two groups of animals, resistance to the disordering effects of ethanol correlated with the presence of cardiolipin from the mitochondria of the ethanol-fed animals (20). In addition to the changes in the lipids per se, a decrease of 18% in the phospholipid/protein ratio in the mitochondrial membranes was found (13), reminiscent of some of the observations on protonophore-resistant bacterial strains. Cunningham and co-workers (see, e.g., reference 74) have shown changes in a number of mitochondrial membrane-associated activities in ethanol-fed animals. A particularly interesting finding in connection with the current discussion is an increased sensitivity of the F_1F_0-ATPase to dissociation, together with a decrease in the total mitochondrial ATPase activity.

Extensive studies have been conducted on the effects of alcohols on membrane-associated transport and catalytic functions, on the composition of the membrane lipids, and on the lipid/protein ratio of the membrane in bacteria (see, e.g., reference 42). The results are complex, depending upon the alcohol used, the species and its normal membrane composition, and whether the species has an inherently high tolerance to alcohol, e.g., *Zymomonas mobilis*. Addition of ethanol to *E. coli* led to a decrease in saturated fatty acid (palmitic acid) levels and an increase in unsaturated fatty acid (primarily vaccenic acid) levels, apparently resulting from inhibition of palmitic acid synthesis (42); the lipid/protein ratio was, however, decreased (41), as was observed in the uncoupler-resistant mutant UV6.

The studies of ethanol tolerance and fusion-resistant mutants, as well as the proposed mechanisms for decoupling by certain membrane-active agents, all highlight the importance of the membrane lipid-protein interactions in energy-coupling phenomena. This is also one of the emerging features in studies of the uncoupler-resistant mutants in bacteria. It represents a divergence from the classical chemiosmotic view. In that view, the specific properties of the membrane lipids and their interactions with the catalytic proteins might very well affect activities and conceivably influence gating phenomena. However, they were unimportant with respect to energy coupling per se, except insofar as they had an impact on the proton and compensatory ion permeability of the bulk membrane. By contrast, the studies reviewed here are more consistent with an important role for the membrane lipids—their individual physical properties, their packing properties, and their physical state in the membrane—in the path that protons take during energy coupling and in the susceptibility of coupling to membrane-active agents. Future studies will further define the interplay of the proteins and lipids in the membrane, and the important elements of specificity therein, in relation to energy coupling. It may well be that specific organisms or individuals are adapted to particular variations in environment such that genetic differences in their coupling membrane lipids allow a relative use of alternative proton pathways that maximizes energy coupling in that environment.

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